TISSUE OXYGENATION MEASUREMENTS

FIELD OF THE INVENTION

This invention relates to the fields of medicine, radiology, and diagnostic imaging. More particularly, the invention relates to a method for assessing oxygenation in a tissue.

BACKGROUND OF THE INVENTION

Hypoxia due to inadequate blood supply, low oxygen levels in the blood, and/or high tissue demand is a significant cause of morbidity and mortality throughout the world. Accordingly, there is a great need in clinical medicine for methods of detecting low oxygen levels in tissues. Current methods of detecting hypoxia *in vivo* include angiography and experimental magnetic resonance imaging (MRI)-based methods. Angiography utilizes x-ray technology and a contrast solution to visualize blood flow through the arteries. Although effective, angiography is also invasive and can cause undesired side-effects from use of contrast agents.

Experimental MRI-based for measuring tissue oxygenation are advantageous over angiography because they are more direct (measuring oxygen levels directly rather than by inference from blood flood) and less invasive. Moreover experimental MRI-based methods offer very good resolution. For example, "BOLD" MRI can be used to image hemoglobin oxygenation within intact red blood cells. To obtain good resolution, however, current experimental MRI methods of detecting tissue oxygen levels require the use of specialized equipment (e.g., that generate very high magnetic fields) not often found in most clinical diagnostic facilities. An MRI-based method for directly determining tissue oxygenation using standard clinical MRI equipment would therefore be of great benefit.

SUMMARY

The invention relates to the discovery that a blood substitute bound to a water-soluble polymer can serve as an MRI contrast agent in MRI-based methods for assessing tissue oxygenation in a subject using MRI equipment (e.g., standard clinical MRI equipment). The

contrast agent allows resolution of tissue oxygen levels without requiring the use of very high magnetic fields produced in experimental MRI methods. The contrast agent includes a blood substitute conjugated to a molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., a water-soluble polymer).

In the experiments described below, polyethylene glycol (PEG) derivatives of bovine hemoglobin (pegylated Hb or PEG-Hb) were used as the contrast agent. The hemoglobin portion of this agent provides MR contrast because at the deoxygenated state the constituent iron is paramagnetic and provides lower relaxation times. Pegylation of Hb alters the hydration of the Hb molecule and thereby changes the proton magnetic resonance (MR) signals produced upon oxygenation/deoxygenation during MRI. In the examples described below, the nuclear MR characteristics of PEG-Hb in oxygenated and deoxygenated states were examined. Substantial changes in the T_1 and T_2 values depending on the state of oxygenation were observed. For example, using the index of the reciprocal change in T_2, there was an approximately 34% change in that value, a percentage change indicative of a successful imaging agent.

PEG-Hb is advantageous for use as a contrast agent for a number of reasons. First, it has been evaluated thoroughly for use as a blood substitute and has proven to be stable and non-toxic. Second, it is highly accessible to water protons compared to Hb contained in erythrocytes. Moreover, the polyethylene glycol portion of the molecule facilitates the diffusion of the water molecules. Third, because the PEG-Hb molecule is relatively small compared to an erythrocyte, it can pass through the walls of the capillaries into the interstitial space allowing the tissue itself to be analyzed. Thus the rate at which PEG-Hb is deoxygenated as it moves into the interstitial space can be detected using MRI. In this manner, hypoxic (ischemic) and normoxic tissue can be distinguished.

In a preferred embodiment, the invention provides a method for assessing oxygenation in a tissue, the method comprising introducing to the tissue a contrast agent comprising surface modified hemoglobin; placing the tissue in a magnetic field and irradiating the tissue with radio frequency energy; and determining spin-lattice and spin-spin relaxation times of water protons associated with oxygenated and deoxygenated states of the hemoglobin in the tissues; thereby,
determining the oxygenation in the tissue. The method further comprises comparing the spin-

lattice or the spin-spin relaxation times of the oxygenated and the deoxygenated states of the hemoglobin to assess tissue oxygenation. In addition, extrapolation of the spin-lattice and spin-spin relaxation times of water protons to generate an image correlated with tissue oxygenation levels.

In another preferred embodiment, a molecule is used to modify the surface of hemoglobin comprising any one or more: polyethylene glycol (PEG), s-nitrosylated PEG (SNO-PEG); nucleophilic PEGs, carboxyl PEGs, electrophilically activated PEGs, sulfhydryl-selective PEGs, heterofunctional PEGs, biotin PEGs, vinyl derivatives, PEG silanes or PEG phospholipids. The hemoglobin surface can also comprise molecules comprising pyridoxal phosphate, alpha-carboxymethyl, or omega-carboxymethoxyl polyoxyethylene (POE).

In another preferred embodiment, the contrast agent provides a ρ_1 of at least about $0.5x10^6$ $(s*mM)^{-1}$ or a ρ_2 of at least about $1x10^6$ $(s*mM)^{-1}$ at a field strength of about 1.5 T on a per particle basis.

In a preferred embodiment, the invention provides a method of determining oxygenation in a patient, the method comprising administering to the patient a contrast agent comprising surface modified hemoglobin; subjecting the patient to a magnetic field and irradiating the patient with radio frequency energy; and determining spin-lattice and spin-spin relaxation times of water protons associated with oxygenated and deoxygenated states of the hemoglobin in the patient; thereby, determining the oxygenation levels in the patient.

In accordance with the invention, the method further comprises the step of comparing the spin-lattice or the spin-spin relaxation times of the oxygenated and the deoxygenated states of the hemoglobin to assess oxygenation. In addition, the method comprises the step of extrapolating the spin-lattice and spin-spin relaxation times of water protons to generate an image correlated with tissue oxygenation levels.

In a preferred embodiment, the hemoglobin is surface modified by a molecule comprising any one or more: polyethylene glycol (PEG), s-nitrosylated PEG (SNO-PEG); nucleophilic PEGs, carboxyl PEGs, electrophilically activated PEGs, sulfhydryl-selective PEGs, heterofunctional PEGs, biotin PEGs, vinyl derivatives, PEG silanes or PEG phospholipids. Optionally, the hemoglobin surface comprises molecules comprising pyridoxal phosphate, alpha-carboxymethyl, or omega-carboxymethoxyl polyoxyethylene (POE). The surface modifying molecule, such as for example, PEG, can be attached to the

hemoglobin with a linker. The linker can be an alkyl, amide, carbamate or phenyl group. The linker can also be selected as an unsaturated aliphatic or aromatic C_1 to C_6 moiety.

In another preferred embodiment, the contrast agent provides a ρ_1 of at least about $0.5x10^6$ $(s*mM)^{-1}$ or a ρ_2 of at least about $1x10^6$ $(s*mM)^{-1}$ at a field strength of about 1.5 T on a per particle basis.

In another preferred embodiment, the contrast agent has a lower P50 value (greater affinity for O_2) as compared to normal hemoglobin.

In another preferred embodiment, the contrast agent has a higher P50 value (lower affinity for oxygen) as compared to normal hemoglobin.

In accordance with the invention the contrast agent can have varying P50 levels depending on the tissue or *in vivo* location to be imaged. As described, these affinities for oxygen are varied by selecting the molecule that modifies the surface of the hemoglobin.

In one embodiment of the invention, the contrast agent is targeted to a desired destination; however, this is not the case for all purposes. For example, the contrast agents of the invention are useful in blood pool contexts or in the gastrointestinal tract where specific localization is unnecessary. However, the particles may also be targeted to specific organs or types of tissue, including fibrin clots, liver, pancreas, neurons, or any tissue characterized by particular cell surface or other ligand-binding moieties. In order to effect this targeting, a suitable ligand is coupled to the contrast agent directly or indirectly. An indirect method is described in U.S. Pat. No. 5,690,907, incorporated herein by reference. In this method, the lipid/surfactant layer of a nanoparticle is biotinylated and the targeted tissue is coupled to a biotinylated form of its specific binding ligand. The biotinylated nanoparticle then reaches its target through the mediation of avidin which couples the two biotinylated components.

In accordance with the invention the contrast agent optionally comprises proteins, glycoproteins, and polysaccharides.

In another preferred embodiment, the method is diagnostic of injury to a patient. The injury can be traumatic such as those injuries suffered by mechanical means, for example, auto accidents, gun shot wounds, concussions and the like. The method is also diagnostic of injuries sustained from biological onslaughts such as tumors, lesions caused by biological agents such as bacteria, virus, chemicals and the like. In particular, the method is useful for diagnosing tumors such as breast cancer, prostate cancer, testicular, lung and the like. The size of the tumor is determined by the necrotic site which would have low to no oxygenation levels.

In another preferred embodiment, the invention provides a contrast agent wherein the inert core is a blood substitute, perfluorocarbon compound or a mixture of fluorocarbons and oils. The contrast agent further comprises a paramagnetic ion, wherein the paramagnetic ion is selected from the group consisting of scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, molybdenum, ruthenium, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, and ytterbium.

Preferably, the surfactant coating is cross-linked. The surfactant coating preferably comprises at least one compound selected from the group consisting of a natural phospholipid, a synthetic phospholipid, a fatty acid, a cholesterol, a lysolipid, a sphingomyelin, a tocopherol, a glucolipid, a stearylamine, a cardiolipin, a lipid with an ether-linker fatty acid, a lipid with an ester linked fatty acid, a polymerized lipid, and a polyethylene glycol-conjugated lipid.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the present specification, including definitions, will control. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The particular embodiments discussed below are illustrative only and not intended to be limiting.

Other aspects of the invention are described *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is pointed out with particularity in the appended claims. The above and further advantages of this invention may be better understood by referring to the following description taken in conjunction with the accompanying drawings, in which:

FIG. 1 is a pair of graphs showing the differences in T_1 and T_2 relaxation times for the oxygenated (Oxy-Hb) and deoxygenated (Deoxy-Hb) forms of PEG-Hb as a function of time for two different samples.

DETAILED DESCRIPTION

A preferred method of the invention for assessing oxygenation in a tissue (e.g., in a subject) utilizes PEG-HB as an MRI contrast agent. The invention is particularly advantageous for analyzing tissue oxygenation to, for example, diagnose ischemia and/or infarction. The invention can be also be used to analyze areas of dangerously low oxygenation in tissue such as the brain (e.g., after strokes or at risk of extension), heart, (e.g., after a heart attack or at risk of infarction), bowel (e.g., mesenteric ischemia), or limbs (e.g., for claudication) and to detect the presence of tumors (which are hypoxic). In addition to diagnosing, the imaging methods can be used to direct decision making for revascularization procedures. They can also be used to examine cerebral or myocardial perfusion during states of variable activity or perfusion.

Definitions

To facilitate understanding of the invention set forth in the disclosure that follows, a number of terms are defined below.

The term "hemoglobin" refers generally to the protein contained within red blood cells that transports oxygen. Each molecule of hemoglobin has 4 subunits, 2 α-chains and 2 β-chains, which are arranged in a tetrameric structure. Each subunit also contains one heme group, which is the iron-containing center that binds oxygen. Thus, each hemoglobin molecule can bind 4 oxygen molecules.

The term "modified hemoglobin" includes, but is not limited to, hemoglobin altered by a chemical reaction such as intra- and inter-molecular cross-linking, genetic manipulation, polymerization, and/or conjugation to other chemical groups (e.g., polyalkylene oxides, for example polyethylene glycol, or other adducts such as proteins, peptides, carbohydrates, synthetic polymers and the like). In essence, hemoglobin is "modified" if any of its structural

or functional properties have been altered from its native state. As used herein, the term "hemoglobin" by itself refers both to native, unmodified, hemoglobin, as well as modified hemoglobin.

The term "surface-modified hemoglobin" is used to refer to hemoglobin described above to which chemical groups such as dextran or polyalkylene oxide have been attached, most usually covalently. The term "surface modified oxygenated hemoglobin" refers to hemoglobin that is in the "R" state when it is surface modified.

The term "stroma-free hemoglobin" refers to hemoglobin from which all red blood cell membranes have been removed.

The term "methemoglobin" refers to an oxidized form of hemoglobin that contains iron in the ferric state and cannot function as an oxygen carrier.

The term "MalPEG-Hb" refers to hemoglobin to which malemidyl-activated PEG has been conjugated. Such MalPEG may be further referred to by the following formula:

$$Hb\text{--}(S\text{--}Y\text{--}R\text{--}CH_2\text{ --}CH_2\text{ --}[O\text{--}CH_2\text{ --}CH_2]_n\text{ --}O\text{--}CH_3)_m \quad \text{Formula I}$$

where Hb refers to tetrameric hemoglobin, S is a surface thiol group, Y is the succinimido covalent link between Hb and Mal-PEG, R is an alkyl, amide, carbamate or phenyl group (depending on the source of raw material and the method of chemical synthesis), $[O\text{--}CH_2\text{--}CH_2]_n$ are the oxyethylene units making up the backbone of the PEG polymer, where n defines the length of the polymer (e.g., MW=5000), and $O\text{--}CH_3$ is the terminal methoxy group. PHP and POE are two different PEG-modified hemoglobin.

The term "perfluorocarbons" refers to synthetic, inert, molecules that contain fluorine atoms, and that consist entirely of halogen (Br, F, Cl) and carbon atoms. In the form of emulsions, they are under development as blood substances, because they have the ability to dissolve many times more oxygen than equivalent amounts of plasma or water.

The term "plasma expander" refers to any solution that may be given to a subject to treat blood loss.

The term "oxygen-carrying component" refers broadly to a substance capable of carrying oxygen in the body's circulatory system and delivering at least a portion of that oxygen to the tissues. In preferred embodiments, the oxygen-carrying component is modified hemoglobin.

The term "oxygen affinity" refers to the avidity with which an oxygen carrier such as hemoglobin binds molecular oxygen. This characteristic is defined by the oxygen equilibrium curve which relates the degree of saturation of hemoglobin molecules with oxygen (Y axis) with the partial pressure of oxygen (X axis). The position of this curve is denoted by the value, P50, the partial pressure of oxygen at which the oxygen carrier is half-saturated with oxygen, and is inversely related to oxygen affinity. Hence the lower the P50, the higher the oxygen affinity. The oxygen affinity of whole blood (and components of whole blood such as red blood cells and hemoglobin) can be measured by a variety of methods known in the art. (See, e.g., Winslow et al., *J. Biol. Chem.* 252(7):2331-37 (1977)). Oxygen affinity may also be determined using a commercially available HEMOX™ TM Analyzer (TCS Scientific Corporation, New Hope, Pa.). (See, e.g., Vandegriff and Shrager in "Methods in Enzymology" (Everse et al., eds.) 232:460 (1994)).

The term "oxygenation" refers to partial pressure of oxygen that is normally found in the body. It can range from approximately 20 torr to 100 torr. The term "deoxygenation" refers to the partial pressure of oxygen that is less than normal values, that is about 15 torr. Oxygen in the body is transported in the blood from the lungs, where the partial pressure of oxygen (pO_2) is relatively high (approximately 100 torr), to the tissues, where the partial pressure of oxygen is much lower (typically 20 torr). In the lungs, hemoglobin becomes nearly saturated with oxygen such that 98% of the oxygen-binding sites are occupied. When hemoglobin moves to the tissues, the saturation level drops to 32%. Thus, a total of 98 - 32 = 66% of the potential oxygen-binding sites contribute to oxygen transport.

The term "relaxation" refers to the electromagnetic radiation released from hydrogen nuclei, following irradiation. This is determined by the relaxation times. Two types of relaxation times can be measured. T_1 is the time for the magnetic distribution to return to 63% of its original distribution longitudinally with respect to the magnetic field and the relaxivity, ρ_1, is its reciprocal. T_2 measures the time wherein 63% of the distribution returns to the ground state transverse to the magnetic field. Its reciprocal is the relaxivity index ρ_2. In general, the relaxation times and relaxivities will vary with the strength of the magnetic field; this is most pronounced in the case of the longitudinal component. Thus, a desirable characteristic of any contrast agents is to provide the signal with an enhanced relaxivity both for ρ_1 and ρ_2. The present invention provides such contrast agents.

The term "mixture" refers to a mingling together of two or more substances without the occurrence of a reaction by which they would lose their individual properties; the term

"solution" refers to a liquid mixture; the term "aqueous solution" refers to a solution that contains some water and may also contain one or more other liquid substances with water to form a multi-component solution; the term "approximately" refers to the actual value being within a range, e.g. 10%, of the indicated value.

The term "polyethylene glycol" refers to liquid or solid polymers of the general chemical formula $H(OCH_2CH_2)_nOH$, where n is greater than or equal to 4. Any PEG formulation, substituted or unsubstituted, can be used.

As used herein, "cancer" refers to all types of cancer or neoplasm or malignant tumors found in mammals, including, but not limited to: leukemias, lymphomas, melanomas, carcinomas and sarcomas. Examples of cancers are cancer of the brain, breast, pancreas, cervix, colon, head and neck, kidney, lung, non-small cell lung, melanoma, mesothelioma, ovary, sarcoma, stomach, uterus and Medulloblastoma. As used herein, the terms "cancer," "neoplasm," and "tumor," are used interchangeably and in either the singular or plural form, refer to cells that have undergone a malignant transformation that makes them pathological to the host organism. Primary cancer cells (that is, cells obtained from near the site of malignant transformation) can be readily distinguished from non-cancerous cells by well-established techniques, particularly histological examination. The definition of a cancer cell, as used herein, includes not only a primary cancer cell, but any cell derived from a cancer cell ancestor. This includes metastasized cancer cells, and *in vitro* cultures and cell lines derived from cancer cells. When referring to a type of cancer that normally manifests as a solid tumor, a "clinically detectable" tumor is one that is detectable on the basis of tumor mass; e.g., by procedures described herein.

The terms "patient" or "individual" are used interchangeably herein, and refers to a mammalian subject to be treated, with human patients being preferred. In some cases, the methods of the invention find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

As used herein, the term "infectious agent" refers to an organism wherein growth/multiplication leads to pathogenic events in humans or animals. Examples of such agents are: bacteria, fungi, protozoa and viruses.

Examples of other uses, for diagnosis by the invention include but not limited to other injuries: As used herein, the term "injury or neural injury" is intended to include a damage which directly or indirectly affects the normal functioning of the CNS. For example, the injury can be damage to retinal ganglion cells; a traumatic brain injury; a stroke related injury; a cerebral aneurism related injury; a spinal cord injury, including monoplegia, diplegia, paraplegia, hemiplegia and quadriplegia; a neuroproliferative disorder or neuropathic pain syndrome. Examples of CNS injuries or disease include TBI, stroke, concussion (including post-concussion syndrome), cerebral ischemia, neurodegenerative diseases of the brain such as Parkinson's disease, Dementia Pugilistica, Huntington's disease and Alzheimer's disease, Creutzfeldt-Jakob disease, brain injuries secondary to seizures which are induced by radiation, exposure to ionizing or iron plasma, nerve agents, cyanide, toxic concentrations of oxygen, neurotoxicity due to CNS malaria or treatment with anti-malaria agents, and other CNS traumas.

As used herein, the term "stroke" is art recognized and is intended to include sudden diminution or loss of consciousness, sensation, and voluntary motion caused by rapture or obstruction (e.g. by a blood clot) of an artery of the brain.

As used herein, the term "Traumatic Brain Injury" is art recognized and is intended to include the condition in which, a traumatic blow to the head causes damage to the brain, often without penetrating the skull. Usually, the initial trauma can result in expanding hematoma, subarachnoid hemorrhage, cerebral edema, raised intracranial pressure (ICP), and cerebral hypoxia, which can, in turn, lead to severe secondary events due to low cerebral blood flow (CBF).

The meaning of other terminology used herein should be easily understood by someone of reasonable skill in the art.

The below described preferred embodiments illustrate adaptations of the invention. Nonetheless, from the description of these embodiments, other aspects of the invention can be made and/or practiced based on the description provided below.

The Nature of Oxygen Delivery and Consumption

Although the successful use of the compositions and methods of the present invention do not require comprehension of the underlying mechanisms of oxygen delivery and consumption, basic knowledge regarding some of these putative mechanisms may assist in understanding the discussion that follows. It has generally been assumed that the capillaries

are the primary conveyors of oxygen to the tissue. However, regarding tissue at rest, current findings indicate that there is approximately an equipartition between arteriolar and capillary oxygen release. That is, hemoglobin in the arterial system is believed to deliver approximately one third of its oxygen content in the arteriolar network and one-third in the capillaries, while the remainder exits the microcirculation via the venous system.

The arteries themselves are sites of oxygen utilization. For example, the artery wall requires energy to effect regulation of blood flow through contraction against vascular resistance. Thus, the arterial wall is normally a significant site for the diffusion of oxygen out of the blood. The rate of oxygen consumption by the vascular wall, i.e., the combination of oxygen required for mechanical work and oxygen required for biochemical synthesis, can be determined by measuring the gradient at the vessel wall. See, e.g., Winslow, et al., in "Advances in Blood Substitutes" (1997), Birkhauser, ed., Boston, Mass., pages 167-188. Present technology allows accurate oxygen partial pressure measurements in a variety of vessels. The measured gradient is directly proportional to the rate of oxygen utilization by the tissue in the region of the measurement. Such measurements show that the vessel wall has a baseline oxygen utilization which increases with increases in inflammation and constriction, and is lowered by relaxation.

The vessel wall gradient is inversely proportional to tissue oxygenation. Vasoconstriction increases the oxygen gradient (tissue metabolism), while vasodilation lowers the gradient. Higher gradients are indicative of the fact that more oxygen is used by the vessel wall, while less oxygen is available for the tissue. The same phenomenon is believed to be present throughout the microcirculation.

Magnetic resonance imaging (MRI) has become a useful tool for diagnosis and for research. The current technology relies on detecting the energy emitted when the hydrogen nuclei in the water contained in tissues and body fluids returns to a ground state subsequent to excitation with a radio frequency. Observation of this phenomenon depends on imposing a magnetic field across the area to be observed, so that the distribution of hydrogen nuclear spins is statistically oriented in alignment with the magnetic field, and then imposing an appropriate radio frequency. This results in an excited state in which this statistical alignment is disrupted. The decay of the distribution to the ground state can then be measured as an emission of energy, the pattern of which can be detected as an image. While the above described process is theoretically possible, it turns out that the relaxation rate of the relevant hydrogen nuclei, left to their own devices, is too slow to generate detectable amounts

of energy, as a practical matter. In order to remedy this, the area to be imaged is supplied with a contrast agent, generally a strongly paramagnetic metal, which effectively acts as a catalyst to accelerate the decay, thus permitting sufficient energy to be emitted to create a detectable bright signal. To put it succinctly, contrast agents decrease the relaxation time and increase the reciprocal of the relaxation time--i.e., the "relaxivity" of the surrounding hydrogen nuclei.

Two types of relaxation times can be measured. T_1 is the time for the magnetic distribution to return to 63% of its original distribution longitudinally with respect to the magnetic field and the relaxivity ρ_1, is its reciprocal. T_2 measures the time wherein 63% of the distribution returns to the ground state transverse to the magnetic field. Its reciprocal is the relaxivity index ρ_2. In general, the relaxation times and relaxivities will vary with the strength of the magnetic field; this is most pronounced in the case of the longitudinal component. Thus, a desirable characteristic of any contrast agents is to provide the signal with an enhanced relaxivity both for ρ_1 and ρ_2. The present invention provides such contrast agents.

Contrast Agent

The invention provides a contrast agent including a blood substitute (i.e., an oxygen carrying molecule suitable for administration to an animal subject) conjugated to a molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., a water-soluble polymer). A preferred contrast agent is one whose MR imaging characteristics differ between oxygenated and deoxygenated states. Any suitable blood substitute and any suitable molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., water-soluble polymer) may be used. As examples, suitable blood substitutes include Hb, modified Hb (e.g., cross-linked, polymerized, pegylated), synthetic non-Hb-based agents, fluorocarbon compounds, and perfluorocarbon moieties. Suitable blood substitutes (e.g., Hb) can be obtained commercially. Naturally occurring blood substitutes may be isolated from animal hosts, e.g., mice, rats, rabbits, goats, sheep, pigs, horses, cattle, dogs, cats, and primates such as monkeys, apes, and human beings. A number of different blood substitutes are described in D.R. Spahn, *Critical Care* 3:R91-R92, 1999. A non-exhaustive list of suitable water-soluble polymers includes PEG, dextran, albumin as well as combinations thereof.

In a preferred embodiment, the surface of the contrast agent comprises cross-linked surface molecules. The core agent/particle can be hemoglobin, nanoparticles, polymers and the like. The cross-linked molecules can be any type of surfactant that can be cross linked, for example polymers, biomolecules and the like.

In one embodiment, the surface is coated with a lipophilic material and the tether is anchored into the coating through a hydrophobic moiety such as one or more aliphatic hydrocarbon chains. In one preferred embodiment, the particles themselves can be described generally as nanoparticles having an inert core surrounded by a coating to which any desired materials can be coupled. In the agent of the invention, these materials can include a chelate containing a paramagnetic ion.

With respect to these preferred particles, the inert core can be a vegetable, animal or mineral oil, but is preferably a fluorocarbon compound--perfluorinated or otherwise rendered additionally inert. Mineral oils include petroleum derived oils such as paraffin oil and the like. Vegetable oils include, for example, linseed, safflower, soybean, castor, cottonseed, palm and coconut oils. Animal oils include tallow, lard, fish oils, and the like. Many oils are triglycerides.

Fluorinated liquids are particularly useful as cores. These include straight chain, branched chain, and cyclic hydrocarbons, preferably perfluorinated. Some satisfactorily fluorinated, preferably perfluorinated organic compounds useful in the particles of the invention themselves contain functional groups. However, perfluorinated hydrocarbons are preferred. The nanoparticle core may comprise a mixture of such fluorinated materials. Typically, at least 50% fluorination is desirable in these inert supports. Preferably, the inert core has a boiling point of above 20°C., more preferably above 30°C., still more preferably above 50°C., and still more preferably above about 90°C.

Thus, the perfluoro compounds that are particularly useful in the above-described nanoparticle aspect of the invention include partially or substantially or completely fluorinated compounds. Chlorinated, brominated or iodinated forms may also be used. A detailed list of compounds useful as nanoparticle cores is included below.

With respect to the coating on the nanoparticles in this aspect, the relatively inert core is provided with a lipid/surfactant coating that will serve to anchor the desired moieties to the nanoparticle itself. If an emulsion is to be formed, the coating typically should include a surfactant. Typically, the coating will contain lecithin type compounds which contain both polar and non-polar portions as well as additional agents such as cholesterol. Typical

materials for inclusion in the coating include lipid surfactants such as natural or synthetic phospholipids, but also fatty acids, cholesterols, lysolipids, sphingomyelins, tocopherols, glucolipids, stearylamines, cardiolipins, a lipid with ether or ester linked fatty acids, polymerized lipids, and lipid conjugated polyethylene glycol. Other surfactants are commercially available.

The foregoing may be mixed with anionic and cationic surfactants.

Fluorochemical surfactants may also be used. These include perfluorinated alcohol phosphate esters and their salts; perfluorinated sulfonamide alcohol phosphate esters and their salts; perfluorinated alkyl sulfonamide alkylene quaternary ammonium salts; N,N-(carboxyl-substituted lower alkyl) perfluorinated alkyl sulfonamides; and mixtures thereof. As used with regard to such surfactants, the term "perfluorinated" means that the surfactant contains at least one perfluorinated alkyl group. A detailed list of surfactants, including fluorinated surfactants that can be used in the coating, is found below.

Typically, the lipids/surfactants are used in a total amount of 0.01-5% by weight of the nanoparticles, preferably 0.1-1% by weight. In one embodiment, lipid/surfactant encapsulated emulsions can be formulated with cationic lipids in the surfactant layer that facilitate the adhesion of nucleic acid material to particle surfaces. Cationic lipids include DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoium chloride; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; and DOTB, 1,2-dioleoyl-3-(4'-trimethyl-ammonio)butanoyl-sn-glycerol may be used. In general the molar ratio of cationic lipid to non-cationic lipid in the lipid/surfactant monolayer may be, for example, 1:1000 to 2:1, preferably, between 2:1 to 1:10, more preferably in the range between 1:1 to 1:2.5 and most preferably 1:1 (ratio of mole amount cationic lipid to mole amount non-cationic lipid, e.g., DPPC). A wide variety of lipids may comprise the non-cationic lipid component of the emulsion surfactant, particularly dipalmitoylphosphatidylcholine, dipalmitoylphosphatidyl-ethanolamine or dioleoylphosphatidylethanolamine in addition to those previously described. In *lieu* of cationic lipids as described above, lipids bearing cationic polymers such as polyamines, e.g., spermine or polylysine or polyarginine may also be included in the lipid surfactant and afford binding of a negatively charged therapeutic, such as genetic material or analogues there of, to the outside of the emulsion particles.

In addition to the above-described preferred embodiment, a multiplicity of other particulate supports may be used in carrying out the method of the invention. In other embodiments, for example, the particles may be liposomal particles. The literature describing

various types of liposomes is vast and well known to practitioners. As the liposomes themselves are comprised of lipid moieties, the above-described lipids and surfactants are applicable in the description of moieties contained in the liposomes themselves. These lipophilic components can be used to couple to the chelating agent in a manner similar to that described above with respect to the coating on the nanoparticles having an inert core. Micelles are composed of similar materials, and this approach to coupling desired materials, and in particular, the chelating agents applies to them as well. Solid forms of lipids may also be used.

In another example, proteins or other polymers can be used to form the particulate carrier. These materials can form an inert core to which a lipophilic coating is applied, or the chelating agent can be coupled directly to the polymeric material through techniques employed, for example, in binding affinity reagents to particulate solid supports. Thus, for example, particles formed from proteins can be coupled to tether molecules containing carboxylic acid and/or amino groups through dehydration reactions mediated, for example, by carbodiimides. Sulfur-containing proteins can be coupled through maleimide linkages to other organic molecules which contain tethers to which the chelating agent is bound. Depending on the nature of the particulate carrier, the method of coupling so that an offset is obtained between the dentate portion of the chelating agent and the surface of the particle will be apparent to the ordinarily skilled practitioner.

In all cases, to serve as MRI contrast agents, the particles are coupled through the required spacer to a chelator in which a transition metal is disposed. Typical chelators include porphyrins, ethylenediaminetetraacetic acid (EDTA), diethylenetriamine-N,N,N',N",N"-pentaacetate (DTPA), 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7 (ODDA), 16-diacetate, N-2-(azol-1(2)-yl)ethyliminodiacetic acids, 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid (DOTA), 1,7,13-triaza-4,10,16-trioxacyclo-octadecane-N,N',N"-triacetate (TTTA), tetraethylene glycols,1,5,9-triazacyclododecane-N,N',N",-tris(methylenephosphonic acid (DOTRP),N,N',N"-trimethylammonium chloride (DOTMA) and analogues thereof.

Examples of typical core components, referred to above, include, but not limited to: perfluorocarbon compounds which may be employed are perfluorotributylamine (FC47), perfluorodecalin (PP5), perfluoromethyldecalin (PP9), perfluorooctylbromide, perfluorotetrahydrofuran (FC80), perfluroether (PID), [(CF3).sub.2 CFOCF2 (CF2) 2 CF2 OCF(CF3) 2]perfluoroether (PIID) [(CF3) 2 CFOCF2 (CF2) 6 CF2 OCF(CF3) 2], perfluoroetherpolymer (Fomblin Y/01), perfluorododecane, perfluorobicyclo[4.3.0.] nonane,

perfluorotritrimethylbicyclohexane, perfluorotripropylamine, perfluoroisopropyl cyclohexane, perfluoroendotetrahydrodicyclopentadiene, perfluoroadamantane, perfluoroexotetrahydrodicyclopentadiene, perfluorbicyclo[5.3.0.]decane, perfluorotetramethylcyclohexane, perfluoro-1-methyl-4-isopropylcyclohexane, perfluoro-n-butylcyclohexane, perfluorodimethylbicyclo[3.3.1.]nonane, perfluoro-1-methyl adamantane, perfluoro-1-methyl-4-t butylcyclohexane, perfluorodecahydroacenapthane, perfluorotrimethylbicyclo[3.3.1.]nonane, perfluoro-1-methyl adamantane, perfluoro-1-methyl-4-t butylcyclohexane, perfluorodecahydroacenaphthene, perfluorotrimethylbicyclo[3.3.1.]nonane, perfluoro-nundecane, perfluorotetradecahydrophenanthrene, perfluoro-1,3,5,7-tetramethyladamantane, perfluorododecahydrofluorene, perfluoro-1-3-dimethyladamantane, perfluoro-n-octylcyclohexane, perfluoro-7-methyl bicyclo[4.3.0.]nonane, perfluoro-p-diisopropylcyclohexane, perfluoro-m-diisopropylcyclohexane, perfluoro-4-methyloctahydroquinolidizine, perfluoro-N-methyldecahydroquinoline, F-methyl-1-oxadecalin, perfluorooctahydroquinolidizine, perfluoro 5,6-dihydro-5-decene, perfluoro-4,5-dihydro-4-octene, perfluorodichlorooctane and perfluorobischlorobutyl ether, perfluorooctane, perfluorodichlorooctane, perfluoro-n-octyl bromide, perfluoroheptane, perfluorodecane, perfluorocyclohexane, perfluoromorpholine, perfluorotripropylamine, perfluortributylamine, perfluorodimethylcyclohexane, perfluorotrimethylcyclohexane, perfluorodicyclohexyl ether, perfluoro-n-butyltetrahydrofuran, and compounds that are structurally similar to these compounds. Chlorinated perfluorocarbons, such as chloroadamantane and chloromethyladamantane as described in U.S. Pat. No. 4,686,024 may be used. Such compounds are described, for example in U.S. Pat. Nos. 3,962,439; 3,493,581, 4,110,474, 4,186,253; 4,187,252; 4,252,824; 4,423,077; 4,443,480; 4,534,978 and 4,542,147.

Commercially available surfactants are Pluronic F-68, Hamposyl™ L30 (W.R. Grace Co., Nashua, N.H.), sodium dodecyl sulfate, Aerosol 413 (American Cyanamid Co., Wayne, N.J.), Aerosol 200 (American Cyanamid Co.), Lipoproteol.TM. LCO (Rhodia Inc., Mammoth, N.J.), Standapol™ SH 135 (Henkel Corp., Teaneck, N.J.), Fizul™ 10-127 (Finetex Inc., Elmwood Park, N.J.), and Cyclopol™ SBFA 30 (Cyclo Chemicals Corp., Miami, Fla.); amphoterics, such as those sold with the trade names: Deriphat™ 170 (Henkel Corp.), Lonzaine™ JS (Lonza, Inc.), Niranol™ C2N-SF (Miranol Chemical Co., Inc., Dayton, N.J.), Amphoterge™ W2 (Lonza, Inc.), and Amphoterge™ 2WAS (Lonza, Inc.); non-ionics, such as those sold with the trade names: Pluronic™ F-68 (BASF Wyandotte, Wyandotte,

Mich.), Pluronic™ F-127 (BASF Wyandotte), Brij™ 35 (ICI Americas; Wilmington, Del.), Triton™ X-100 (Rohm and Haas Co., Philadelphia, Pa.), Brij™52 (ICI Americas), Span™ 20 (ICI Americas), Generol™ 122 ES (Henkel Corp.), Triton™ N-42 (Rohm and Haas Co.), Triton™ N-101 (Rohm and Haas Co.), Triton™ X-405 (Rohm and Haas Co.), Tween™ 80 (ICI Americas), Tween™ 85 (ICI Americas), and Brij™ 56 (ICI Americas) and the like.

Also included may be egg yolk phospholipids, alkylphosphoryl choline or alkylglycerolphosphoryl choline surfactants, and specific examples of these such as 1,2-dioctylglycero-3-phosphoryl choline, 1,2-ditetradecylglycero-3-phosphoryl choline, 1,2-dihexadecylglycero-3-phosphoryl choline, 1,2-dioctadecylglycero-3-phosphorylcholine, 1-hexadecyl-2-tetradecylglycero-3-phosphoryl choline, 1-octadecyl-2-tetradecylglycero-3-phosphoryl choline, 1-tetradecyl-2-octadecylglycero-3-phosphoryl choline, 1-hexadecyl-2-octadecylglycero-3-phosphoryl choline, 1-2-dioctadecylglycero-3-phosphoryl choline, 1-octadecyl-2-hexadecylglycero-3-phosphoryl choline, 1-tetradecyl-2-hexadecylglycero-3-phosphoryl choline, 2,2-ditetradecyl-1-phosphoryl choline ethane and 1-hexadecyltetradecylglycero-3-phosphoryl choline.

Suitable perfluorinated alcohol phosphate esters include the free acids of the diethanolamine salts of mono- and bis(1H,1H,2H,2H-perfluoroalkyl)phosphates. The phosphate salts, available under the trade name "Zonyl RP" (E.I. Dupont de Nemours and Co., Wilmington, Del.), are converted to the corresponding free acids by known methods. Suitable perfluorinated sulfonamide alcohol phosphate esters are described in U.S. Pat. No. 3,094,547. Suitable perfluorinated sulfonamide alcohol phosphate esters and salts of these include perfluoro-n-octyl-N-ethylsulfonamidoethyl phosphate, bis(perfluoro-n-octyl-N-ethylsulfonamidoethyl)phosphate, the ammonium salt of bis(perfluoro-n-octyl-N-ethylsulfonamidoethyl)phosphate,bis(perfluoro-decy l-N-ethylsulfonamidoethyl)-phosphate and bis(perfluorohexyl-N ethylsulfonamidoethyl)-phosphate. The preferred formulations use phosphatidylcholine, derivatized-phosphatidylethanolamine and cholesterol as the aqueous surfactant.

Suitable paramagnetic metals include a lanthanide element of atomic numbers 58-70 or a transition metal of atomic numbers 21-29, 42 or 44, i.e., for example, scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, molybdenum, ruthenium, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, and ytterbium, most preferably Gd(III), Mn(II), iron, europium and/or dysprosium.

According to the invention, the chelating moiety is coupled to the particle through a spacer or tether which may be an aliphatic chain, a peptide, a polyethylene glycol polymer, or any suitable spacing molecule. One end of the spacer is bound, preferably covalently, to the dentate portion of the chelating agent; the other is anchored to the particle. The coupling to the particle can be covalent or the spacer may be anchored through ionic bonding, hydrogen bonding or van der Waals forces. When the particle surface comprises a lipid surface, particularly preferred anchoring moieties are the hydrocarbon side chains of phosphatides or other di-substituted glycerol derivatives.

By appropriately coupling the chelating agents, substantial numbers of chelators and paramagnetic ions can be coupled to the particles. Typically, the particles will be coupled to at least 10,000 chelators and/or paramagnetic ions, preferably 20,000 chelators and/or paramagnetic ions, more preferably 50,000 chelators and/or paramagnetic ions, more preferably at least 70,000 chelators and/or paramagnetic ions and more preferably at least 100,000 chelators and/or paramagnetic ions.

As set forth above, the tether is such that an offset is obtained sufficient to confer the relaxivity values described above, and spacing the paramagnetic ion from the surface of the particle as described.

While the particles of the invention are required to comprise a multiplicity of paramagnetic ions coupled through chelating agents, additional components may also be coupled to these particles. Especially advantageous for use of the contrast agents in some applications of MRI is the inclusion of a ligand which is a specific binding partner for a target on a tissue desired to be imaged. It may also be desirable to provide a biologically active substance and this may he included as well.

Thus, in addition to the chelated paramagnetic metal ion, the particles may also be coupled to ligands for targeting and/or biologically active molecules. It is possible also to include among the components coupled to the particles bearing the chelated paramagnetic ion, radionuclides for use in treatment or diagnosis.

The precise process for preparation of the contrast agents of the invention is variable, and depends on the nature of the particulate carrier and the choice of tether or spacer molecules. As described above, solid particles which contain reactive groups can be coupled directly to the tether or spacer; lipid-based particles such as oil emulsions, solid lipids, liposomes, and the like, can include lipophilic materials containing reactive groups which may covalently, then, be coupled to linking moieties which bear the dentate portion of the

chelating agent. In one particularly preferred embodiment, the process involves mixing a liquid fluorocarbon compound that forms the core of a nanoparticle and the components of a lipid/surfactant coating for that particle in an aqueous suspension, microfluidizing, and, if desired, harvesting and sizing the particles. The components to be coupled can be included in the original mixture by virtue of their initial coupling to one or more components of the lipid/surfactant coating, or the coupling to additional moieties can be conducted after the particles are formed.

In the examples described below, pegylated bovine Hb was used as the contrast agent. Hb is preferred as a blood substitute because the relaxation times of oxygenated and deoxygenated Hb differ, resulting in an oxygen-sensitive contrast agent. Bovine Hb is a beneficial source because it is inexpensive, readily available, may be easily modified and has increased oxygen carrying capacity. PEG, a water-soluble polymer, is preferred for use in the invention as a molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation because it imparts an improved interaction between Hb and water molecules in the tissue. PEG is a gel-like substance that facilitates the diffusion of the water molecules. The PEG polymer stabilizes the Hb protein and the resulting conjugate is non-toxic, highly accessible to water protons, and able to penetrate through interstitial space. As PEG-Hb is relatively small compared to the erythrocyte, PEG-Hb molecules are capable of passing through the walls of capillaries, thereby increasing the number of locations in which the PEG-Hb may be used as a contrast agent. In addition, the PEG polymer minimizes antigenicity and does not change the blood chemistry or the function of the cardiovascular system.

PEG-Hb may be obtained from any commercial source. Otherwise, the Hb may be purified directly from a source and then attached to a PEG-based polymer. Alternatively, the Hb may be obtained from a commercial source and then attached to a PEG-based polymer by methods known in the art. One such method is set forth in U.S. Patent No. 5,650,388, which is incorporated herein by reference. PEG-Hb is also commercially available. If obtained from a commercial source, PEG-Hb should be checked for the presence of contaminants such methemoglobin. If present, the PEG-Hb should be purified before use to ensure proper results. For example, contaminants such as methemoglobin could confound the results of MRI tests.

In alternative embodiments, the PEG-Hb of the present invention may include derivatives in which the PEG moiety or the Hb protein is modified. The PEG-Hb may be a

Hb protein conjugated to PEG that has been modified with another moiety, for instance, s-nitrosylated PEG (SNO-PEG). Other useful PEG derivatives include, but are not limited to, nucleophilic PEGs, carboxyl PEGs, electrophilically activated PEGs, sulfhydryl-selective PEGs, heterofunctional PEGs, biotin PEGs, vinyl derivatives, PEG silanes and PEG phospholipids

Alternatively, the PEG-Hb may be a Hb protein conjugated to PEG in which the Hb has been modified with another moiety, for instance, pyridoxal phosphate, resulting in a pyridoxylated PEG-Hb derivative. In yet another embodiment, the PEG-Hb protein may be conjugated to an alpha-carboxymethyl, omega-carboxymethoxyl polyoxyethylene (POE), resulting in a POE conjugated PEG-Hb derivative. In each of these embodiments, the PEG-Hb is safe and non-toxic. In one embodiment, the PEG-Hb derivative of the invention is a PEG polymer conjugated to a Hb protein.

PEG-hemoglobin has a reduced diffusion constant, transferred O_2 in a manner very similar to that of native red blood cells (McCarthy, M. R., K. D. Vandegriff, and R. M. Winslow, "The role of facilitated diffusion in oxygen transport by cell-free hemoglobin: Implications for the design of hemoglobin-based oxygen carriers," *Biophysical Chemistry* 92: 103-117 (2001)). Oxygen affinity would be expected to play a role in its facilitated diffusion by hemoglobin in the plasma space, since the change in saturation from the hemoglobin to the vessel wall is a determinant of the diffusion gradient of the hemoglobin itself.

Oxygen affinity of cell-free hemoglobin may play an additional role in the regulation of vascular tone, since the release of O_2 to vessel walls in the arterioles will trigger vasoconstriction (Lindbom, L., R. Tuma, and K. Arfors, "Influence of oxygen on perfusion capillary density and capillary red cell velocity in rabbit skeletal muscle," *Microvasc Res* 19: 197-208 (1980)). In the hamster skinfold, the P O_2 in such vessels is in the range of 20-40 Torr, where the normal red cell oxygen equilibrium curve is steepest (Intaglietta, M., P. Johnson, and R. Winslow, "Microvascular and tissue oxygen distribution," *Cardiovasc Res* 32: 632-643 (1996)). Thus from a theoretical point of view, it may be important for the P50 of cell-free hemoglobin to be lower than that of red cells (i.e., higher O_2 affinity), in order to prevent release of O_2 in arteriolar regulatory vessels.

In preferred embodiments, the oxygen carrier (i.e., the oxygen-carrying component) is a hemoglobin-based oxygen carrier, or HBOC. The hemoglobin may be either native (unmodified); subsequently modified by a chemical reaction such as intra- or inter-molecular cross-linking, polymerization, or the addition of chemical groups (e.g., polyalkylene oxides,

or other adducts); or it may be recombinantly engineered. Human alpha- and beta-globin genes have both been cloned and sequenced. Liebhaber, et al., *P.N.A.S.* 77: 7054-7058 (1980); Marotta, et al., *J. Biol. Chem.* 353: 5040-5053 (1977) (beta-globin cDNA). In addition, many recombinantly produced modified hemoglobins have now been produced using site-directed mutagenesis, although these "mutant" hemoglobin varieties were reported to have undesirably high oxygen affinities. See, e.g., Nagai, et al., P.N.A.S., 82: 7252-7255 (1985).

The present invention is not limited by the source of the hemoglobin. For example, the hemoglobin may be derived from animals and humans. Preferred sources of hemoglobin for certain applications are humans, cows and pigs. In addition, hemoglobin may be produced by other methods, including chemical synthesis and recombinant techniques.

In addition to the aforementioned sources of hemoglobin, it has recently been found that horse hemoglobin has certain advantages as the oxygen carrying component in the compositions of the present invention. One advantage is that commercial quantities of horse blood are readily available from which horse hemoglobin can be purified. For use in the present invention, the hemoglobin, when altered by for example, pegylation, changes the proton MR signals upon oxygenation and/deoxygenation which is used for detection by MR.

Hemoglobin is known to exhibit autooxidation when it reversibly changes from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) or methemoglobin form. When this happens, molecular oxygen dissociates from the oxyhemoglobin in the form of a superoxide anion (O^{2-}). This also results in destabilization of the heme-globin complex and eventual denaturation of the globin chains. The PEG-Hb conjugates described herein exhibit very low rates of autooxidation. When measured as a rate of oxidation, this value should be as low as possible (i.e., 0.2% per hour of total hemoglobin, more preferably 0.1% per hour of total hemoglobin, at room temperature for at least 3 hours, and more preferably at least 10 hours.

In a preferred embodiment, the oxygen-carrying component is modified hemoglobin. A preferred modification to hemoglobin is "surface-modification," i.e. covalent attachment of chemical groups to the exposed amino acid side chains on the hemoglobin molecule. Modification is carried out principally to increase the molecular size of the hemoglobin, most often by covalent attachment of polymeric moieities such as synthetic polymers, carbohydrates, proteins and the like. Generally, synthetic polymers are preferred.

Suitable synthetic hydrophilic polymers include, *inter alia*, polyalkylene oxide, such as polyethylene oxide ($(CH_2CH_2O)_n$), polypropylene oxide ($(CH(CH_3)\ CH_2O)_n$) or a

polyethylene/polypropylene oxide copolymer ($(CH_2CH_2O)_n$ --$(CH(CH_3)CH_2O)_n$). Other straight, branched chain and optionally substituted synthetic polymers that would be suitable in the practice of the present invention are well known in the medical field.

The preferred chemical group attached to the hemoglobin is polyethylene glycol (PEG), because of its pharmaceutical acceptability and commercial availability. PEGs are polymers of the general chemical formula $H(OCH_2CH_2)_nOH$, where n is generally greater than or equal to 4. PEG formulations are usually followed by a number that corresponds to their average molecular weight. For example, PEG-200 has an average molecular weight of 200 and may have a molecular weight range of 190-210. PEGs are commercially available in a number of different forms, and in many instances come preactivated and ready to conjugate to proteins.

Although in many respects the performance of surface modified hemoglobins is independent of the linkage between the hemoglobin and the modifier (e.g. PEG), more rigid linkers can be used, such as unsaturated aliphatic or aromatic C_1 to C_6 linker substituents may enhance the manufacturing and/or characteristics of the conjugates when compared to those that have more flexible and thus deformable modes of attachment.

The number of PEGs to be added to the hemoglobin molecule may vary, depending on the size of the PEG. However, the molecular size of the resultant modified hemoglobin should be sufficiently large to avoid being cleared by the kidneys to achieve the desired half-life.

Although the invention is focused on the combination of Hb and PEG, any suitable combination of blood substitute and any molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., water-soluble polymer) may be used as a contrast agent in methods of the invention.

In other preferred embodiments, the contrast agent can be selectively rendered more detectable within a tissue, structure (e.g. lymphatics, tumor) by exposure to a particular treatment or energy. Moreover, the selected contrast agent may be imaged using other than traditional detection procedures. For example, contrast agents that are traditionally used in conjunction with ultrasound detection methods (i.e., microbubble agents) may, in the context of the present invention, be used with magnetic resonance visualization methods. That is, the contrast agent may be imaged using ultrasonic energy and the infusion of intact, detectable contrast agents monitored through magnetic resonance. While any imaging agent possessing

the requisite properties may be employed, preferred embodiments of the invention comprise the use of ultrasound contrast agents and/or magnetic resonance imaging. agents.

The contrast agents of the invention are not limited to PEG and Hb. Other contrast agents contemplated for use in the present invention may be formulated, for example, from lipids, polymeric materials, proteins, and the like. The lipids, polymers, and/or proteins may be natural, synthetic or semi-synthetic. It should be noted that for medical uses, the selected contrast agent should be biocompatible or not be physiologically deleterious or injurious to biological functions, and which will not result in any degree of unacceptable toxicity, including allergenic responses and disease states. For example, ultimately, components comprising the contrast agent may decay wherein the components will preferably be released into the blood either as dissolved particles or gas or as submicron droplets of condensed liquid. It will be understood that gases will primarily be removed from the body through lung respiration or through a combination of respiration and other metabolic pathways including the reticuloendothelial system.

A preferred synthetic non-hemoglobin based contrast agent may comprise a microbubble preparation wherein the microbubbles are associated with an MRI agent such as a paramagnetic material. A number of contrast agents may be employed in the practice of the present invention, including droplets, bubbles, microbubbles, polymer particles, microspheres, microballoons, microcapsules, suspensions, emulsions, and the like. Examples of microbubbles that can be used in the practice of the present invention are those that are free, or are surrounded or comprise an elastic or rigid shell, wall, or membrane. As employed herein, the term "shell" (used interchangeably with the terms, "wall" or "membrane") is used to refer to the material surrounding or defining a microbubble, whether it be a surfactant, another film forming liquid, a solid or semisolid outer layer, and the like. The walls may be concentric or otherwise. The shell can be formulated from lipids (i.e., phospholipids), natural and synthetic polymeric materials, proteinaceous materials, carbohydrates, sacchirides, and the like. In addition, the shells may be in the form of a monolayer or bilayer, and the mono- or bilayer may be used to form one or more mono- or bilayers. In the case of more than one mono- or bilayer, the mono- or bilayers may be concentric, if desired. Preferably, lipids may be used to form a unilamellar microbubble (comprised of one monolayer or bilayer), an oligolamellar microbubble (comprised of about two or about three monolayers or bilayers) or a multilamellar microbubble (comprised of more than about three monolayers or bilayers). Similarly, the microbubbles prepared from polymers or proteins may comprise one or more

walls or membranes, concentric or otherwise. The walls or membranes of microbubbles prepared from lipids, polymers, or proteins may be substantially solid (uniform), or they may be porous or semi-porous.

When referring to the pressure of dissolved gas in a liquid, the more familiar term "pressure" may be used interchangeably with "tension." "Gas osmotic pressure" is more fully defined below, but in a simple approximation can be thought of as the difference between the partial pressure of a gas inside a contrast agent and the pressure or tension of that gas (either in a gas phase or dissolved in a liquid phase) outside of the contrast agent, when the membrane is permeable to the gas. More precisely, it relates to differences in gas diffusion rates across a membrane.

It will be appreciated that the contrast agents utilized in the present methods should have a lifetime sufficient to enable them to persist for the time period during which images and/or measurements are taken. In one preferred embodiment, the contrast agents are adapted to return a signal at a frequency different from the frequency of the ultrasonic pulse emitted by the transducer, such as a harmonic frequency of the ultrasonic pulse. That is, the contrast agents are adapted for harmonic imaging such as is described in U.S. Pat. No. 5,540,909 which is incorporated herein by reference in its entirety. Yet, it must be emphasized that, while the present invention may often be described in the context of the preferred embodiments, the invention is not limited to the use of such formulations.

The microbubble contrast agents preferably contain a gas and/or vapor (or precursor thereof). When referring to a "gas," it will be understood that mixtures of gases together having the requisite properties fall within the definition, except where the context otherwise requires. A "vapor," on the other hand, is the gaseous phase of a material that is a liquid at ambient temperature and pressure, but that has an appreciable vapor pressure at the relevant temperature, e.g., body temperature. The gas and/or vapor may provide the contrast agent with enhanced imaging capabilities, such as reflectivity of ultrasound, particularly in connection with microbubble compositions in which the gas is entrapped within the microbubble. Those skilled in the art will appreciate that the term "gas" as used herein shall be held to mean gases, gas mixtures or vapors unless otherwise specified.

Fluorocarbon or fluorinated gases or vapors are particularly preferred as osmotic or stabilizing agents for microbubble preparations. The term "fluorocarbon" is used herein in its broadest sense and includes fully fluorinated compounds (perfluorocarbons) as well as partially fluorinated hydrocarbon materials (fluorochemicals or fluorinated compounds),

including unsubstituted chains or those substituted with another halogen such as Br, Cl, or I or another substituent, such as O, OH, S, NO, and the like. For example, sulfur hexafluoride would be considered a fluorocarbon gas for the purposes of the present invention and may be used to provide stabilized microbubble preparations in accordance with the teachings herein. In selected embodiments, microbubbles useful with the present invention may contain materials that can change state from a gas to a liquid or solid at body temperature, (generally from about 35.5°C. to about 40°C.), and at useful pressures (generally about 1-2 atm). Similarly, fluorocarbons or other compounds that are not gases at room or body temperature can be used, provided that they have sufficient vapor pressure, preferably at least about 10-20 Torr, and more preferably 30, 40, 50 or 100 Torr at body temperature, or more preferably at least about 150 or 200 Torr.

In particular, substances possessing suitable solubility and/or vapor pressure criteria for the formation and use of microbubbles in accordance with the invention include fluoroheptanes, fluorocycloheptanes, fluoromethylcycloheptanes, fluorohexanes, fluorocyclohexanes, fluoropentanes, fluorocyclopentanes, fluoromethylcyclopentanes, fluorodimethylcyclopentanes, fluoromethylcyclobutanes, fluorodimethylcyclobutanes, fluorotrimethylcyclobutanes, fluorobutanes, fluorocyclobutanse, fluoropropanes, fluoroethers, fluoropolyethers, fluorotriethylamines, and the like. Particularly preferred embodiments of the present invention employ microbubbles comprising perfluorohexanes, perfluoropentanes, perfluorobutanes, perfluoropropanes, sulfur hexafluoride, and the like. One particular preferable class of compatible compounds comprises flouroethers. Other useful gases or vapors comprise Freon 113, methylene chloride, Freon 12 (dichlorodifluoromethane), Freon 11 (trichloromonofluoromethane), butanes, pentanes, hexanes, propane, methane, ethane, and the like.

Whichever gases or osmotic agents are ultimately selected, it will be appreciated that microbubbles comprising mixtures of gases and/or vapors may be used with the disclosed methods as can microbubbles comprising pure gases. For example, both mixtures of fluorocarbon gases (i.e. fluorohexane and fluorobutane) and fluorocarbon gases mixed with nonfluorocarbon compounds (i.e. fluoropentane and nitrogen) can form particularly stable microbubbles. It will further be appreciated that several types of gas or vapor are compatible with either microbubble configuration, i.e. they are useful as a component of a mixture or in a pure state.

In this regard, mixtures of gases and/or vapors may be used to form particularly long lasting contrast agents. This is because contrast agents of a primary modifier gas such as air or nitrogen (including fluorocarbon gases) saturated with a selected perfluorocarbon osmotic agent can grow rather than shrink when exposed to air dissolved in a liquid due to the osmotic pressure exerted by the perfluorocarbon gas or vapor. Preferably, the osmotic agent is relatively impermeable to the contrast agent film and thus remains inside the contrast agent. Air or other gases inside the contrast agent are diluted by the perfluorocarbon, which acts to slow the air diffusion flux out of the contrast agent. This gas osmotic pressure is proportional to the concentration gradient of the perfluorocarbon vapor across the contrast agent film, the concentration of air surrounding the contrast agent, and the ratio of the contrast agent film permeability to air and to perfluorocarbon.

Further, as disclosed in U.S. Pat. No. 5,315,997, gases and perfluorocarbon vapors have magnetic susceptibilities substantially different from tissues and blood. Therefore, microbubble contrast agents comprising fluorinated compounds will cause changes in the local magnetic fields present in tissues and blood during MRI. As such, the aforementioned microbubble contrast agents may also be used for magnetic resonance visualization. Other exemplary MRI agents, which may be used with the present invention comprise paramagnetic and supraparamagnetic macromolecular compounds or particulates that may be associated with microbubbles (i.e. on the membrane) or mixed with a microbubble contrast agent. Examples of such imaging agents are to be found in U.S. Pat. Nos. 4,675,173 and 4,849,210, each of which is incorporated herein by reference. With respect to paramagnetic compounds, gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA), and transition metal ions of iron and manganese may be used in conjunction with the disclosed invention, particularly when attached to a larger molecule such as human serum albumin, dextran or polylysine.

It will be appreciated that those of ordinary skill in the art can readily determine other compounds that would perform suitably that do not meet both the solubility and vapor pressure criteria, described above. Rather, it will be understood that certain compounds can be considered outside the preferred range in either solubility or vapor pressure, if such compounds compensate for the aberration in the other category and provide a superior insolubility in water or high vapor pressure or affinity to dissolve in the surfactant used.

It will further be understood that other components can be associated with useful contrast agent formulations. For example, osmotic agents and stabilizers (described herein), chelators, surfactants, buffers, viscosity modulators, air solubility modifiers, salts, sugars, and

the like, can be added to fine tune the contrast agent suspensions for maximum life and contrast enhancement effectiveness. Such considerations as sterility, isotonicity, and biocompatibility may govern the use of such conventional additives to injectable compositions. The use of such agents will be understood to those of ordinary skill in the art and the specific quantities, ratios, and types of agents can be determined empirically without undue experimentation. Such components associated with useful contrast agents can be in addition to, or instead of the materials (lipids, polymer, protein, gas, vapor, liquid, and the like, described herein) which comprise the contrast agent. In addition, these components can be associated inside, on or outside the contrast agent. Thus, with respect to microbubbles, such components can be within the void, part of the shell or membrane, or part of the solution which surrounds the microbubble.

In other preferred embodiments, synthetic non-hemoglobin based contrast agents will be elastic or deformable or capable of being elastic under certain conditions, e.g., temperature, pH, light, application of energy (e.g., ultrasound, and the like), and the like. As employed herein, the term "elastic" refers to the ability of contrast agents employed in the present invention to be non-rigid and/or to alter their shape, for example, to pass through an opening that is smaller than the diameter of the contrast agent.

In contrast to the elastic contrast agents described above, it may be desirable, in certain circumstances, to formulate contrast agents from substantially impermeable materials such as polymer materials, including, for example, polymethyl methacrylate. This would generally result in the formation of contrast agents which may be substantially impermeable and relatively inelastic and even brittle. In embodiments involving diagnostic imaging, for example, ultrasound, contrast media which comprise such vesicles with limited elasticity would generally not provide the desirable reflectivity nor the ability to adequately gain access to the lumen of the lymphatic vessel. However, by increasing the power output on ultrasound or by applying another energy source, the vesicle can be made to rupture, thereby causing acoustic emissions which can be detected by an ultrasound transducer, can be deformed in such a way as to gain access to the lumen of the lymph vessel, or alternatively, release its contents that can then gain access to the lumen of the lymph vessel. Further, the contents of the vesicle can have diagnostic or therapeutic function that can be released in an extravascular site of interest such as a tumor, a lymph node, or any normal or abnormal region of a patient.

Various combinations of the materials comprising or surrounding the contrast agents may be used to modify the relaxation behavior of the medium or to alter properties such as the viscosity, osmolarity, stability, sterility, isotonicity, biocompatibility, imageability, brightness, and the like. For example, the gas and vapor filled contrast agents used in the present invention may be controlled according to size, solubility and heat stability by choosing from among the various additional or auxiliary stabilizing materials described herein. These materials can affect these parameters of the contrast agents, especially contrast agents formulated from lipids, not only by their physical interaction with the membranes, but also by their ability to modify the viscosity and surface tension of the surface of the gas and gaseous precursor filled vesicle.

Accordingly, the gas and gaseous precursor filled contrast agents contemplated for use in the present invention may be favorably modified and further stabilized, for example, by the addition of one or more of a wide variety of (a) viscosity modifiers, including, for example, carbohydrates and their phosphorylated and sulfonated derivatives; polyethers, preferably with molecular weight ranges between about 400 and about 100,000; di- and trihydroxy alkanes and their polymers, preferably with molecular weight ranges between about 200 and about 50,000; and the like; (b) emulsifying and/or solubilizing agents including, for example, acacia, cholesterol, diethanolamine, glyceryl monostearate, lanolin alcohols, lecithin, mono-and di-glycerides, mono-ethanolamine, oleic acid, oleyl alcohol, poloxamer, for example, poloxamer 188, poloxamer 184, and poloxamer 181, polyoxyethylene 50 stearate, polyoxyl 35 castor oil, polyoxyl 10 oleyl ether, polyoxyl 20 cetostearyl ether, polyoxyl 40 stearate, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, propylene glycol diacetate, propylene glycol monostearate, sodium lauryl sulfate, sodium stearate, sorbitan mono-laurate, sorbitan mono-oleate, sorbitan mono-palmitate, sorbitan monostearate, stearic acid, trolamine, emulsifying wax, and the like; (c) suspending and/or viscosity-increasing agents, including, for example, acacia, agar, alginic acid, aluminum mono-stearate, bentonite, magma, carbomer 934P, carboxymethylcellulose, calcium and sodium (and/or sodium 12), carrageenan, cellulose, dextran, gelatin, guar gum, locust bean gum, veegum, hydroxyethyl cellulose, hydroxypropyl methylcellulose, magnesium-aluminum-silicate, Zeolites(r), methylcellulose, pectin, polyethylene oxide, povidone, propylene glycol alginate, silicon dioxide, sodium alginate, tragacanth, xanthan gum, alpha -d-gluconolactone, glycerol, mannitol, and the like; (d) synthetic suspending agents, such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), polyvinyl alcohol

(PVA), polypropylene glycol (PPG), polysorbate, and the like; (e) tonicity raising agents which stabilize and add tonicity, including, for example, sorbitol, mannitol, trehalose, sucrose, propylene glycol, glycerol, and the like; and other similar materials.

The contrast agent can be used alone, or in combination with additional diagnostic, therapeutic or other agents. Such other agents include excipients such as coloring materials. As employed herein, the term "diagnostic agents" refers to detectable agents, in addition to or other than the contrast agents described herein, useful in diagnostic methods, e.g., MR agents, CT agents, ultrasound agents, optical imaging agents, dyes, and the like. As employed herein, the term "bioactive agents" refers to biologically active agents, e.g., therapeutic compounds. In this respect it will be appreciated that compatible bioactive agents may be selected from the group consisting of analgesics, antibiotics, leukotriene inhibitors or antagonists, antihistamines, antiinflammatories, antineoplastics, anticholinergics, anesthetics, enzymes, steroids, genetic material, viral vectors, antisense agents, proteins, peptides, and the like, and combinations thereof.

In vivo Targeting

In another preferred embodiment, the surface of the particle or agent is modified by cross-linked molecules and/or have a specific ligand for targeting. In a preferred method, the specific ligand itself is coupled directly to the particle, preferably but not necessarily, covalently. Thus, in such "direct" coupling, a ligand which is a specific binding partner for a target contained in the desired location is itself linked to the components of the particle, as opposed to indirect coupling where a biotinylated ligand resides at the intended target. Such direct coupling can be effected through linking molecules or by direct interaction with a surface component. Homobifunctional and heterobifunctional linking molecules are commercially available, and functional groups contained on the ligand can be used to effect covalent linkage. Typical functional groups that may be present on targeting ligands include amino groups, carboxyl groups and sulfhydryl groups. In addition, crosslinking methods, such as those mediated by glutaraldehyde could be employed. For example, sulfhydryl groups can be coupled through an unsaturated portion of a linking molecule or of a surface component; amides can be formed between an amino group on the ligand and a carboxyl group contained at the surface or *vice versa* through treatment with dehydrating agents such as carbodiimides. A wide variety of methods for direct coupling of ligands to components of particles in general and to components such as those found in a lipid/surfactant coating in one

embodiment are known in the art. The foregoing discussion is non-comprehensive. In a specific case which employs aptamers, it may be advantageous to couple the aptamer to the nanoparticle by the use of a cationic surfactant as a coating to the particles.

In one embodiment of the invention, the contrast agent is targeted to a desired destination; however, this is not the case for all purposes. For example, the contrast agents of the invention are useful in blood pool contexts or in the gastrointestinal tract where specific localization is unnecessary. However, the particles may also be targeted to specific organs or types of tissue, including fibrin clots, liver, pancreas, neurons, or any tissue characterized by particular cell surface or other ligand-binding moieties. In order to effect this targeting, a suitable ligand is coupled to the contrast agent directly or indirectly. An indirect method is described in U.S. Pat. No. 5,690,907, incorporated herein by reference. In this method, the lipid/surfactant layer of a nanoparticle is biotinylated and the targeted tissue is coupled to a biotinylated form of its specific binding ligand. The biotinylated nanoparticle then reaches its target through the mediation of avidin which couples the two biotinylated components.

The targeting agent itself may be any molecule which is specific for an intended target. Commonly, such a ligand may comprise an antibody or portion thereof, an aptamer designed to bind the target in question, a known ligand for a specific receptor such as an opioid receptor binding ligand, a hormone known to target a particular receptor, a peptide mimetic and the like. Certain organs are known to comprise surface molecules which bind known ligands; even if a suitable ligand is unknown, antibodies can be raised and modified using standard techniques and aptamers can be designed for such binding.

Antibodies or fragments thereof are preferred targeting agents because of their capacity to be generated to virtually any target, regardless of whether the target has a known ligand to which it binds either natively or by design. Standard methods of raising antibodies, including the production of monoclonal antibodies are well known in the art and need not be repeated here. It is well known that the binding portions of the antibodies reside in the variable regions thereof, and thus fragments of antibodies which contain only variable regions, such as F_{ab}, F_v, and scF_v moieties are included within the definition of "antibodies." Recombinant production of antibodies and these fragments which are included in the definition are also well established. If the imaging is to be conducted on human subjects, it may be preferable to humanize any antibodies which serve as targeting ligands. Techniques for such humanization are also well known.

The precise process for preparation of the contrast agents of the invention is variable, and depends on the nature of the particulate carrier and the choice of tether or spacer molecules. As described above, solid particles which contain reactive groups can be coupled directly to the tether or spacer; lipid-based particles such as oil emulsions, solid lipids, liposomes, and the like, can include lipophilic materials containing reactive groups which may covalently, then, be coupled to linking moieties which bear the dentate portion of the chelating agent. In one embodiment, the process involves mixing a liquid fluorocarbon compound that forms the core of a nanoparticle and the components of a lipid/surfactant coating for that particle in an aqueous suspension, microfluidizing, and, if desired, harvesting and sizing the particles. The components to be coupled can be included in the original mixture by virtue of their initial coupling to one or more components of the lipid/surfactant coating, or the coupling to additional moieties can be conducted after the particles are formed.

The contrast agents may be used without targeting ligands for obtaining images where homing to a site is unnecessary, such as blood pool images. However, where specific organs are to be imaged, targeted forms of the particles are preferred.

Determining An Oxygen Concentration In A Tissue

The invention also provides a method for assessing oxygenation in a tissue. The method includes the steps of providing a tissue, introducing a contrast agent to the tissue, wherein the contrast agent comprises a blood substitute conjugated to a water-soluble polymer, and subjecting the tissue to MRI. Following irradiation of the tissues, electromagnetic radiation is released from hydrogen nuclei in water molecules (i.e., relaxation). In select embodiments, the irradiation is irradiation in the radiofrequency range. The spin-lattice (T_1) and the spin-spin relaxation (T_2) times of the protons are determined. In one variation of the method, the values for T_1 and T_2 are compared for both the oxygenated and deoxygenated states of PEG-Hb to evaluate the oxygenation in tissues. In another variation of this method, the T_1 and T_2 values are utilized by MR software to reconstruct a visual image of oxygenation in a tissue. The method is expected to be useful in assessing tissue oxygenation qualitatively, as well as quantitating oxygenation levels in tissues. The method can be used in many applications, including, for example, the detection of ischemia in tissues. Other MRI techniques, such as "bold" or "fast" MRI, may be used with the contrast agents of the present invention. MRI methods are described in detail in Kuperman,

V., Magnetic Resonance Imaging Physics and Biomedical Applications, Academic Press, 2000. The use of NMR imaging to examine tissue oxygenation is described in R. Vink, *Adv. Exp. Med. Biol.* 316:187-193, 1992 and Ogawa *et al., Proc. Natl. Acad. Sci.* 87:9868-9872, 1990.

Imaging

In a preferred embodiment, the contrast agents are utilized with magnetic resonance (MR) imaging. Methods for imgaing using MR are described in detail in the examples which follow. In other preferred embodiments of the present invention, the contrast agents utilized in the present invention are useful for diagnostic imaging, including, for example, X-ray, X-ray computed tomography (CT) imaging, including CT angiography (CTA) imaging, magnetic resonance angiography (MRA), nuclear medicine, ultrasound (US) imaging, optical imaging, elastography, infrared imaging, microwave imaging, and the like.

For X-ray or computed tomography imaging, the contrast agent should have a different electron density than surrounding tissues (either more or less electron density) to render it visible with these techniques. With respect to contrast agents for CT, it is generally sought to employ agents that will increase electron density in certain areas of a region of the body (positive contrast agents). Suitable electron density is achieved, for example, in compounds with bromine, fluorine or iodine moieties, and in materials comprising or including radiopaque metal atoms. With respect to contrast agents for CT, is also sought to employ agents that will decrease electron density in certain areas of a region of the body (negative contrast agents). Suitable agents can be fat or air or any substance with lower electron density than water.

For MRI, contrast agents which are suitable for use in accordance with invention methods should have adequate nuclear or relaxation properties or susceptibility effect for imaging that are different from the corresponding properties of the tissue being imaged. With respect to synthetic nonhemoglobin-based agents, either an imageable nucleus (such as ^{19}F), radionuclides, diamagnetic, paramagnetic, ferromagnetic, superparamagnetic substances, and the like, can be used with appropriate MRI equipment.

Ultrasound and X-ray imaging, including the use of digital subtraction techniques, may also be utilized according to another embodiment of the present invention. Ultrasound contrast agents can be selected on the basis of density or acoustical properties. Preferably, the contrast agent is echogenic. As employed herein, the term "echogenic" refers to a

contrast agent that may be capable of reflecting or emitting sound waves. Echogenic contrast agents may be particularly useful to alter, for example, the acoustic properties of a lymph tissue, organ or region of a patient, preferably the sentinel lymph node, thereby resulting in improved contrast in diagnostic imaging techniques, such as those described herein. As previously alluded to, microbubble preparations are particularly compatible with ultrasound imaging.

In this respect, suitable contrast agents include Imagent (AFO150), Alliance Pharmaceutical Corp., San Diego, Calif.; AI-700, Acusphere, Inc., Cambridge, Mass., AIP201 by UofV; Albunex and Optison (FS069), both by Molecular Biosystems, Inc., San Diego, Calif.; Echogen and QW7437 both by Sonus Pharmaceuticals Bothell, Wash.; Levovist (SH/TA-508), Echovist and Sonovist (SHU563), all by Schering AG, Berlin, Germany; Aerosomes-DMP115 and MRX115, by ImaRx Pharmaceuticals, Tucson, Ariz.; BR1 and BR14, both by Bracco International B.V., Amsterdam, NL; Quantison and Quantison Depot, both by Andaris, Ltd. Nottingham, GB; and NC100100, Nycomed Imaging AS, Oslo, Norway, and the like. Contrast agents and methods of forming contrast agents usable in the present invention are disclosed in U.S. Pat. Nos. 4,957,656, 5,141,738, 4,657,756, 5,558,094, 5,393,524, 5,558,854, 5,573,751, 5,558,853, 5,595,723, 5,558,855, 5,409,688, 5,567,413, 5,558,856, 5,556,610, 5,578,292, 5,271,928, 5,531,980 5,562,893, 5,837,221, 4,572,203, 4,844,882, 5,552,133, 5,536,489 and 5,558,092, each of which is incorporated herein by reference in its entirety. International applications WO 96/40282, WO 95/01187 and WO 96/40278 further describe compatible contrast agent preparations and are also incorporated herein. Additional suitable contrast agents, as well as their compatible characteristics are described, for example, in Calliada *et al., Eur J. Radio.* (1998) Suppl 2:S157-160, the disclosures of which are hereby incorporated herein by reference in their entirety.

For optical imaging, optically active gases, such as argon or neon, may be incorporated in the present compositions. In addition, optically active materials, for example, fluorescent materials, including porphyrin derivatives, may also be used. Elastography is an imaging technique which generally employs much lower frequency sound, for example, about 60 KHz, as compared to ultrasound (which can involve frequencies of over about 1 MHz). In elastography, the sound energy is generally applied to the tissue and the elasticity of the tissue may then be determined. The lipid based vesicles described herein are preferably highly elastic, and they may increase the local elasticity of tissue to which they are

directed. This increased elasticity may then be detected with elastography. If desired, elastography can be used in conjunction with other imaging techniques, such as MRI and ultrasound.

Subjects

Because subjects from many different species are susceptible to low tissue oxygenation (e.g., ischemia, infarction), the invention is believed to be compatible with many types of animal subjects. Thus one example of the foregoing method includes the step of administering the contrast agent to a subject and then subjecting the subject to MRI. A non-exhaustive exemplary list of animal subjects that might be analyzed includes mammals such as mice, rats, rabbits, goats, sheep, pigs, horses, cattle, dogs, cats, and primates such as monkeys, apes, and human beings. Those animal subjects known to suffer from or suspected to suffer from low tissue oxygenation are preferred for use in the invention. In particular, human patients suffering from or suspected of suffering from low tissue oxygenation are suitable subjects for use in the invention.

Effective Amounts

The contrast agents described above are preferably administered to a mammal (e.g., human) in an effective amount, that is, an amount capable of producing a desirable result in a treated mammal (e.g., being detectable in *in vivo* imaging). Such a therapeutically effective amount can be determined as described below.

Toxicity and therapeutic efficacy of the compositions utilized in methods of the invention can be determined by standard pharmaceutical procedures, using either cells in culture or experimental animals to determine the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Those compositions that exhibit large therapeutic indices are preferred. While those that exhibit toxic side effects may be used, care should be taken to design a delivery system that minimizes the potential damage of such side effects. The dosage of preferred compositions lies preferably within a range that includes an ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

As is well known in the medical and veterinary arts, dosage for any one animal depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, time and route of administration, general health, and other drugs being administered concurrently. It is expected that an appropriate dosage for intravenous administration of PEG-Hb would be in the range of about 3-5% of total blood volume (e.g., approximately 200 ml).

The compositions described above may be administered to animals including human beings in any suitable formulation by any suitable method. For example, a conventional syringe and needle can be used to inject a contrast agent formulation into a subject. Depending on the desired route of administration, injection can be *in situ* (i.e., to a particular tissue or location on a tissue), intramuscular, intravenous, intraperitoneal, or by another parenteral route. Parenteral administration of a contrast agent can be performed, for example, by bolus injection or continuous infusion.

To facilitate delivery of the contrast agent, the contrast agent may be mixed with pharmaceutically acceptable carriers or diluents such as physiological saline or a buffered salt solution. Suitable carriers and diluents can be selected on the basis of mode and route of administration and standard pharmaceutical practice. A description of exemplary pharmaceutically acceptable carriers and diluents, as well as pharmaceutical formulations, can be found in Remington's Pharmaceutical Sciences, a standard text in this field, and in USP/NF. Other substances may be added to the compositions to stabilize and/or preserve the compositions.

Administration of Contrast Agents

Any of the above described contrast agent for imaging regions with low oxygen tension, including systems such as the lymphatic system may be administered to a vertebrate subject, such as a bird or a mammal. As employed herein, the term "lymphatic system" refers to cells, tissues or organs which comprise or are associated with the lymph system, including lymph nodes, lymph vessels, lymph canals, lymph cells, macrophages, injection situs, and the like. Preferably, the vertebrate is a human, and the lymph structure of interest, such as the lymph nodes, lymph vessels, and the like, can be imaged with any of the techniques described herein. This can be useful, e.g., for detecting the lymph nodes, tumors, necrotic regions, and infected regions.

In accordance with a preferred embodiment of the present invention, there are provided methods for identifying the regions of low oxygen tension as described in the examples which follow. Major areas of interest for imaging areas with low oxygen tension to assess blood supply and tissue viability, include regional spread of neoplastic and infectious lesions of the breast, colon and rectum, prostate, ovary, testes, skin cancer, and the like. Major lymph nodes involved in these various lesions include axillary and internal mammary nodes in the chest, and the pararectal, anterior pelvic (obturator), internal iliac (hypogastric), presacral, external and common iliac, and para-aortic nodes. Thus, applications where lymphographic imaging would be useful include, but are not limited to, pathological lesions affecting the major organs of the chest, abdomen and pelvis, as well as the skin, from which the regional and, subsequently, more distant lymphatics can be involved. A preferred method of imaging is MR. However, other methods are also useful. The different modes of visualization are known in the art, as well as suitable modes of administration of contrast agents, are discussed, for example, in Vogl *et al.* (*Acta Radiol.* (1997) Supp 412:47-50), the disclosure of which is hereby incorporated by reference in its entirety.

As those skilled in the art would recognize, administration of the contrast agents described herein, as well as the auxiliary materials, can be carried out in various fashions which are not intravascular, including parenteral. Parenteral administration, which is preferred, includes administration by the following routes: intramuscular, percutaneous, directly in the lymphatic vessel or the lymph node, intraepidermal, intramedullary, intramural or intraparenchymal, interstitially, intraperitoneal, intrathecal, subcutaneous, intrasynovial, transepithelial (including transdermal), dermal, in the tumor or pathologic process itself, and the like. Preferably, the contrast agent is administered by interstitial injection (or other interstitial administration) in the vicinity of the tissue, organ, lymph node and the like, to be imaged, including subcutaneous (under or in the skin) and intraparenchymal (into an organ) injection, but not intraperitoneal injection (into a body cavity). In the case of cancer patients, the contrast agent is preferably injected in proximity to the cancer. The contrast agent can also be injected by a combination of two or more parenteral modes, for example intramuscular, subcutaneous, and in the pathologic process, insuring its accretion in the lymphatic structure of interest. Upon administration, the contrast agent is preferably taken up by the lymphatic system, generally localizing in lymph nodes (preferably the sentinel lymph node) afferent to the uptake site. Thus, preferably, the contrast agent would follow the same route as a metastatic tumor cell would be likely to follow within the lymphatic system.

Suspensions or formulations comprising contrast agents are administered in a manner compatible with the route of administration, the dosage formulation, and in a diagnostic effective amount. It is anticipated that dosages between about 0.1 to about 30 ml of the agent (about 10 micrograms up to about 1 milligram per kilogram of body weight) per day will be used for diagnostic applications. In accordance with a preferred embodiment of the present invention, a small quantity of the contrast agent (e.g., about 0.1 ml/Kg based on the body weight of the vertebrate) is introduced into the animal per injection site, but this can vary depending on the site and the number of injections. Other quantities of the contrast agent, such as from about 0.005 ml/Kg to about 1.0 ml/Kg, are also contemplated for use in the practice of the present invention. Volumes of the contrast agent will normally vary somewhat depending upon the site of injection, the concentration and activity of the preparation, the number of injections to be used, the particular contrast agent employed, the characteristics of the tissue desired, the degree and duration of effect desired, the judgment of the practitioner, as well as properties peculiar to each individual. Moreover, suitable dosage ranges for systemic application depend on the route of administration. Adjustment of these parameters will be conventional for the ordinary skilled clinician. Suitable regimes for initial administration are variable, but are typified by an initial administration followed by repeated doses at one or more intervals.

In addition, the contrast agent may be in the form of a sterile injectable suspension or formulation comprising contrast agents combined with suitable carriers. Suitable carriers include non-toxic parenterally-acceptable sterile aqueous or nonaqueous solutions, suspensions, or emulsions, including the auxiliary or stabilizing materials, surfactants, and the like (each which have been described herein). This suspension may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents, including the auxiliary or stabilizing materials described herein. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate, or the like. They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the formulations, by irradiating the formulations, by heating the formulations, and the like. Sterile injectable

suspensions may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. Buffers, preservatives, antioxidants, and the like can also be incorporated as required.

The imaging agent will normally be administered at a site and by means that insure that it is mobilized and taken up into the system. This will vary with the system to be imaged. Multiple injection sites may be preferable in order to permit proper drainage to the region under investigation. In some cases, injections around the circumference of a tumor or biopsy site is desired. In other cases, injection into a particular sheath or fossa is preferred. Injection into the webs of the fingers or toes is a common mode used to study, for example, peripheral lymphatics. The contrast agent can be administered to the subject either pre-operatively and/or intra-operatively to localize the desired region. It is recognized that the present invention, preferably, allows immediate and real-time identification of the lymph vessel and the sentinel draining node following percutaneous injection of the contrast agent in a region of interest. Administration of the contrast agent does not require significant lead time to reach the region of interest. In addition, additional methodology can be employed to modify or alter the transport of the contrast agent, including massaging the injection site or stimulating flow by exercise to facilitate transport of the contrast agent to the structure of interest.

As discussed, the invention method can be used to visualize damaged tissues, lesions, tumors and the like. For example, to visualize the regions associated with genitourinary cancers or lesions, bilateral deep perianal injection of the contrast agent into the ischiorectal fossa is effective. The patient can be placed in the lithotomy position and about 1 ml of the contrast agent is introduced bilaterally into the ischiorectal fossa, e.g., with a 22 gauge needle, to a depth of about 1.5 inches just lateral to the anal margin, at the 9 and 3 o'clock positions. The patient may also lie on the side if achieving the lithotomy position is not possible. Subcutaneous dorsal pedal injection of about 0.5 ml of the contrast agent may also be made, e.g., using a 23 gauge half-inch needle in the first interdigital spaces bilaterally. In certain cases, such as testicular or prostatic cancer or some cases of rectal carcinoma, intratumoral or peritumoral injection of imaging agents can be effective.

The present method also has applicability in locating the sentinel nodes associated with breast tumor. Images of axillary, subclavian and supraclavicular nodes may be obtained by injecting the contrast agent into and around the tumor and below the skin adjacent to the tumor or the medial surface of the upper arms (ipsilateral and contralateral) of patients with

breast cancer. A unilateral injection can be made in the subcostal site ipsilateral to the tumor, and then repeated later on the contralateral side to observe cross drainage between the ipsilateral and contralateral nodes. Alternatively, for example, for visualization of the internal mammary lymphatics in breast cancer, the contrast agent is injected into the posterior rectus sheath at the insertion of the diaphragm in the subcostal site, using about 1 ml of the contrast agent. By injecting a contrast agent in the vicinity of the tumor, the practitioner will know that the lymph duct involved and leading to the sentinel node will be directed toward the axillary, internal mammary, or supraclavicular chain wherein imaging is effected at appropriate times after each injection.

Another approach is to inject about 0.5 to 1 ml of contrast agent around the areola tissue of the breasts bilaterally, and then imaging the axillary, internal mammary, or supraclavicular chains. In addition to periareolar injection, interdigital administration of the contrast agent may be used for visualization of axillary lymphatics (see, DeLand *et al.*, (1980), *Cancer Res.* 40:2997-3001). Combined interdigital and periareolar administration of the contrast agent can provide increased accuracy to demonstrate increased uptake in affected axillary nodes. Intratumoral injection of the contrast agent can also be performed in patients with breast cancer or melanoma and is a useful mode of administration for certain cases.

Preferably, the contrast agents persist for a sufficient amount of time following administration to allow measurements of the rate of increase in contrast agent levels in the target region and the determination of maximum signal strength. In this respect preferred imaging agents have a half life of at least about 1 minute following administration. More preferably the imaging agents have a half life of at least about 2, 3, 5, 10 or 30 minutes or more following administration. However, those skilled in the art will appreciate that the disclosed invention may be practiced by continuous administration of an imaging agent having any half life including those with half lives on the order of seconds or tens of seconds.

In accordance with yet another embodiment of the present invention, the sentinel node is then removed for evaluation as to the presence or absence of neoplastic or infectious diseases or disorders, including metastasis. Thus, the diagnostic procedure is minimally invasive, as other non-affected regional axillary nodes are not disturbed. When compared with the conventional surgical protocols of removing essentially all regional lymph nodes at the axilla, the minimally invasive aspect of the present methodology immediately becomes apparent.

Imaging Techniques

In a preferred embodiment, the imaging technique is magnetic resonance imaging (MRI). Magnetic resonance imaging (MRI) is used for producing images of the body in a variety of scanning planes such as, for example, axial, coronal, sagittal or orthogonal. MRI employs a magnetic field, radio frequency energy and magnetic field gradients to make images of the body. Similar to CTs, the magnetic resonance imaging techniques which are employed are conventional and are described, for example, in D. M. Kean and M. A. Smith, Magnetic Resonance Imaging: Principles and Applications, (William and Wilkins, Baltimore 1986), and in Rajan, S. S., MRI: A Conceptual Overview (Springer Verlag 1997), the disclosures of which are incorporated by reference herein in their entirety. Contemplated MRI techniques include, but are not limited to, nuclear magnetic resonance (NMR), electronic spin resonance (ESR), and the like.

In accordance with the present invention, any imaging techniques which allow for the monitoring of the infusion of contrast agent into the target region are compatible with the teachings herein. In this regard, all forms of imaging techniques are contemplated in the present invention, including, for example, imaging by X-ray, computed tomography (CT) imaging, including CT angiography (CTA) imaging, magnetic resonance (MR) imaging, magnetic resonance angiography (MRA), nuclear medicine, ultrasound (US) imaging, optical imaging or spectroscopy, elastography, infrared imaging, microwave imaging, and the like. Moreover, the imaging may be combined to provide multiple exposures of the contrast agent following administration. The imaging techniques that are employed are known in the art, and these techniques are described generally in Kopans, D. B. Md., Breast Imaging (Lippincott-Raven Publishers 1998), the disclosure of which is incorporated by reference herein in its entirety.

Computed tomography (CT) is a valuable diagnostic imaging technique for studying various areas of the body. This technique measures the radiodensity (electron density) of matter. CT imaging techniques which are employed are conventional and are described, for example, in Computed Body Tomography, Lee, J. K. T., Sagel, S. S., and Stanley, R. J., eds., 1983, Ravens Press, New York, N.Y., especially the first two chapters thereof entitled "Physical Principles and Instrumentation", and Scroggin, Lippincotts Computer Tomography Review (Lippincott-Raven Publishers 1995), the disclosures of which are incorporated by reference herein in their entirety.

With respect to ultrasound, ultrasonic imaging techniques contemplated for use in the present invention are well known in the art, and are described, for example, in McGahan and Goldberg, Diagnostic Ultrasound: A Logical Approach (Lippincott-Raven Publishers 1998), and in Frederick and Kremkau, Diagnostic Ultrasound: Principles and Instruments, (W B Saunders Co. 1998), the disclosures of which are hereby incorporated herein by reference in their entirety.

Specific ultrasound imaging modes useful with the disclosed invention include harmonic or non-linear imaging, grey scale (B-mode), Doppler (including pulsed wave, Power, flow, color, amplitude, spectral and harmonic), 3-D imaging, gated imaging, and the like. With respect to harmonic imaging, it will be appreciated that the present invention is compatible with wideband harmonic imaging and pulse inversion harmonic imaging. Those skilled in the art will further appreciate that any of these imaging modes may be used to provide the signal levels which, upon processing, can afford the desired values for fluid flow rates and fluid content.

If one desires to use harmonic imaging (an optional embodiment of the present invention), and the ultrasound imaging machine is set to image at a particular frequency, the outgoing waveform supplied to the sonic transducer can be a numerical fraction of the imaging frequency (e.g., 1/2, 2/3, 1/3, and the like) or a whole number or fractional multiple of the imaging frequency (e.g., 2, 3/2, 3, 4, and the like). With any particular combination of contrast agents and excitation frequency, certain harmonics will be dominant. The second harmonic is a common example. Those strongest harmonics are preferred for obvious reasons, although other harmonics or frequencies may be selected for reasons such as preparation of multiple images or elimination of background. Moreover several frequencies, including harmonic and non-harmonic frequencies or some combination thereof, may be simultaneously detected to provide the desired image. That is, in preferred embodiments any frequency other than the interrogation frequency may be used to provide the desired data. Of course, those skilled in the art will appreciate that dominant harmonics can be determined by simple empirical testing of the contrast agent preparation.

To detect the reradiated ultrasound energy generated by the contrast agents, a modified conventional ultrasound scanner system or commercially available harmonic imaging systems can be used. These systems are able to detect or select one or more or all of the new frequencies, or harmonics, radiated by the contrast agents for production of the ultrasound image. In other words, it detects a frequency different from the emitted

frequency. Equipment suitable for harmonic ultrasound imaging is disclosed in Williams *et al.*, WO 91/15999. Many conventional ultrasound imaging devices, however, utilize transducers capable of broad bandwidth operation, and the outgoing waveform sent to the transducer is software controlled. For this reason, reprogramming to emit a waveform different from the one detected is well within the level of skill in the art.

Although harmonic ultrasound imaging is preferred for use in the disclosed methods and systems, other types of ultrasound imaging such as B-mode (gray scale imaging), F-mode (color flow or Doppler imaging) and D-mode (spectral Doppler) are also compatible and within the purview of the instant invention.

In B-mode imaging, the ultrasound system typically transmits a series of beams, along scan lines, steered to scan a desired field of view. The ultrasound system typically steers "receive beams" in a manner that corresponds to the transmit beams. Data returned from each receive beam is communicated to an image display subsystem which reconstructs a two-dimensional gray scale image from the B-mode data and displays it on a console. Such series of pulses down a single line may be identical or may be of equal or unequal frequency or have a near 180 degree phase shift (inverted pulse) to promote the distinction of the contrast agent from the surrounding tissues.

F-mode imaging is accomplished in a manner similar to B-mode imaging, in that the ultrasound system fires and receives a series of beams to scan a field of view. However, since F-mode imaging requires calculation of the velocity of targets, each line is fired and received several times. As with B-mode imaging, the data returned from each firing of each line is used to reconstruct an image on a console. F-mode imaging is often used concurrently with B-mode imaging. For example, the gray scale image reconstructed from a B-mode scan can be superimposed with an F-mode image reconstructed from an F-mode scan of the same field of view or of a lesser included field of view. The F-mode information can be displayed using colors, with different colors indicating different positive or negative flow velocities or turbulence at the part of the B-mode image on which the pixel is superimposed. Because F-mode imaging is intended to provide only qualitative insight into target motion in the patient's body, the ultrasound system's processing of F-mode signals need not have high spatial or velocity resolution either in amplitude or in pixel resolution. However, since an important value of F-mode imaging is to detect flows relative to anatomical structures in the body, it is usually important that the F-mode image be properly registered with the B-mode image on-screen. Since this technique relies on the correlation of signal obtained from one pulse versus

the subsequent pulse, and since vesicles can be destroyed by the first pulse, an F-signal is generated that is not related to motion. This loss of correlation can be shown in a variety of display formats but is typically displayed in color.

In D-mode (spectral Doppler) acquisition, the ultrasound system fires a beam and processes the return signal for a single target. Spectral Doppler information can be obtained by transmitting and receiving either continuous wave (CW) or pulsed wave (PW) ultrasonic energy. In CW Doppler acquisition, for example, Power Doppler (Doppler angiography), the ultrasound receiver continuously receives echoes from all objects within the receiver's area of sensitivity in the body, and cannot isolate information received from any specific range interval. CW Doppler is most useful where the instrument's area of sensitivity can be adjusted, either by physical placement of the probe or by beamforming, or both, to include only the desired target. I n PW Doppler acquisition, the ultrasound instrument receives echoes from individual pulses, the timing of which implies a range interval within the body of the object which produced the echo. A clinician typically selects a range interval within which the target is expected to be located.

In D-mode acquisition, it is desirable to be able to produce detailed quantitative measurements over a very large range of signal levels (dynamic range). D-mode information is processed by the ultrasound system to display either the velocity spectrum of the target, plotted against time, or to provide an audio output carrying similar information. Spectral Doppler acquisition is described in Liv Hatle, M.D. & Bjorn Angelsen, Dr. Techn., "Doppler Ultrasound in Cardiology" (1st ed. 1982) and (2d ed. 1984), incorporated herein by reference in its entirety.

In addition to B-, F- and D-mode acquisition, a fourth mode also exists, known as M-mode, but this is merely a different display modality for data acquired in a manner similar to B- or F-mode acquisition. The requirements for M-mode acquisition are not significantly different from those for B- or F-mode acquisition. Alternatively, or in addition, 3-dimensional ultrasound is also contemplated, wherein 3-D scans require special probes and software to accumulate and render the images.

Additional diagnostic techniques contemplated for use in the present invention are well known in the art, and are described, for example, in Gamsu *et al.*, Diagnostic Imaging Review (W B Saunders Co 1998), the disclosure of which are incorporated by reference herein in its entirety.

In the case of diagnostic applications (such as ultrasound, CT, MRI, and the like), energy, such as ultrasonic energy, may be applied to at least a portion of the patient to image the target tissue. A visible image of an internal region of the patient may be then obtained.

The following examples are offered by way of illustration, not by way of limitation. While specific examples have been provided, the above description is illustrative and not restrictive. Any one or more of the features of the previously described embodiments can be combined in any manner with one or more features of any other embodiments in the present invention. Furthermore, many variations of the invention will become apparent to those skilled in the art upon review of the specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in pertinent part for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

EXAMPLES

Example 1 - Determination of T_1 and T_2 in Oxygenated/Deoxygenated PEG-Hb Samples

Two hundred microliters of samples containing 3% PEG-Hb were prepared. For deoxygenation, 5 mg of dithionite which had been stored in a desiccator under nitrogen gas was added. The sample was then vortexed and nitrogen gas was injected from the top of the tube. To determine T_1 for the samples, an inversion recovery experiment was performed and a CPMG spin echo experiment was conducted to determine T_2. NMR Conditions: 23°C, 7 Tesla, -80 dB attenuation, spatial width: 400, line broadening: 1 Hz, 1000 points, no zero filling.

Figure 1 shows the results of the NMR experiments. After deoxygenation, T_1 values in the first sample (A) increased from an average of 2.29 sec to 2.37. The second sample (B) increased from an average of 2.06 sec to 2.13 sec, resulting in only a 3% increase in the reciprocal values of T_1 for both samples. The differences in the reciprocal values of T_2 are greater than 30%. In the first sample (A), T_2 decreased from an average of 128 msec to 95 msec, which provided a 34% difference in the reciprocal values of T_2. The second sample

(B) decreased from an average of 97 msec to 73 msec reducing the reciprocal values of T_2 to 33%.

Example 2- Target Tissues

Pegylated hemoglobin (or other blood substitute-based oxygenation imaging agents) is expected to be of broad clinical and experimental use. Such agents can be utilized to ascertain areas of dangerously low oxygenation, such as in the brain (e.g, after strokes or at risk of extension), heart (e.g, after heart attacks or at risk of infarction), bowel (e.g., mesenteric ischemia), detect the presence of tumors (which are hypoxic) or limbs (e.g., for claudication). The results of the imaging could be used diagnostically as well as be a tool to direct decision making for revascularization procedures. In experimental research it similarly could be used to study cerebral or myocardial perfusion during states of variable activity or perfusion.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspect, advantages, and modifications are within the scope of the following claims.